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Targeting prostate cancer cells with genetically engineered polypeptide-based micelles displaying gastrin-releasing peptide

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ABSTRACT

In recent years G protein-coupled receptors (GPCRs) have emerged as crucial tumorigenic factors that drive aberrant cancer growth, metastasis and angiogenesis. Consequently, a number of GPCRs are strongly expressed in cancer derived cell lines and tissue samples. Therefore a rational anti-cancer strategy is the design of nano-medicines that specifically target GPCRs to bind and internalise cytotoxic drugs into cancer cells. Herein, we report the genetic engineering of a self-assembling nanoparticle based on elastin-like polypeptide (ELP), which has been fused with gastrin releasing peptide (GRP). These nanoparticles increased intracellular calcium concentrations when added to GRP receptor positive PC-3 prostate cancer cells, demonstrating specific receptor activation. Moreover, GRP-displaying fluorescent labelled nanoparticles showed specific cell-surface interaction with PC-3 prostate cancer cells and increased endocytic uptake. These nanoparticles therefore provide a targeted molecular carrier system for evaluating the delivery of cytotoxic drugs into cancer cells.

1. Introduction

Castrate resistant prostate cancer (CRPC) is the most aggressive form of prostate cancer. The therapeutic effects of chemotherapy drugs and potent anti-androgens like abiraterone and enzalutamide have been very limited and most patients succumbed to advanced malignancy despite this treatment (O'Hanlon Brown and Waxman, 2012). To date, the most significant anti-tumour responses have been achieved in clinical trials with taxane derivatives such as docetaxel (Gollan and Green, 2002) and cabazitaxel (Honary and Zahir, 2013). However, only modest life prolonging effects have been achieved in patients, due to severe clinical complications that include fatigue, immunosuppression, peripheral neuropathy and increased mortality (Gollan and Green, 2002). The outcome could potentially be improved through specific nanoparticle-guided drug delivery into cancer cells, to reduce the exposure of healthy tissue to the drugs.

G-protein-coupled-receptors (GPCR) are the largest family of trans-membrane receptors and many of these receptors are over-expressed in prevalent solid tumours (Cornelio et al., 2007). Therefore, targeting tumour-specific GPCRs is a promising strategy to directly deliver chemotherapeutic drugs into cancer cells. Gastrin-releasing peptide receptor (GRPR) is a member of the bombesin receptor family and is over-expressed in prostate cancer tissue (Bartholdi et al., 1998; Patel et al., 2006). Bombesin-like and bombesin-derived peptides have been covalently bound to paclitaxel for targeted delivery into prostate cancer cells and this strategy enhanced the anti-cancer efficacy (Cornelio et al., 2007; Safavy et al., 2006). In another study, 2-pyrrolino doxorubicin (AN-201) was conjugated to bombesin, which led to improved anti-cancer activities with reduced adverse effects (Cornelio et al., 2007; Stangelberger et al., 2006).

Building on this knowledge we hypothesise that nanoparticles, which expose the human bombesin homologue gastrin releasing peptide (GRP) on the surface, will dock to GRPR expressing cells. This GPCR docking is likely to trigger receptor internalisation and will lead to the delivery of the nanoparticles directly into the endosomal compartments of the target cells (Fig. 1). With this strategy, the efficacy of active cancer cell targeting could be combined with passive targeting by the enhanced permeability and retention (EPR) effect.

Recently, elastin-like polypeptide (ELP) micelles have emerged as potential carriers for anti-cancer compounds (Herrero-Vanrell

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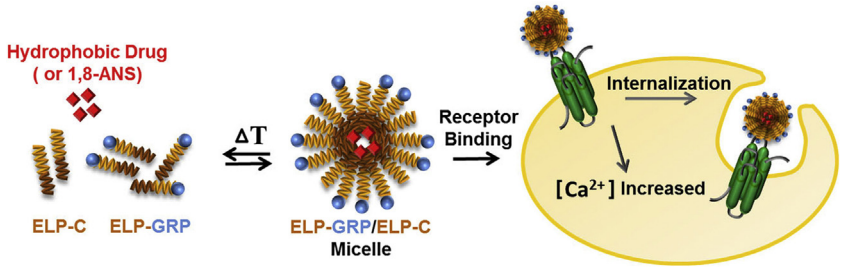

Fig. 1. Targeting prostate cancer cells with ELP-GRP micelles. The self-assembling elastin-like polypeptide (ELP-C) was extended at the C-terminus with 10 amino acids derived from GRP leading to the ELP-GRP fusion protein. Micelles were formed at elevated temperature with GRP (depicted by blue spheres) displayed on the surface. Mixed micelles (ELP-GRP/ELP-C) were shown to have increased stability and successfully induced a specific Ca^{2+} signal in GRPR expressing PC-3 prostate cancer cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2005; MacEwan and Chilkoti, 2014; MacKay et al., 2009; Saxena and Nanjan, 2015; Shi et al., 2013). These proteins consist of repetitive Val-Pro-Gly-X-Gly penta-peptides, where X represents the guest residue, which can be any amino acid except proline (Hassouneh et al., 2012; MacEwan and Chilkoti, 2010). ELPs are routinely engineered as di-block proteins, consisting of hydrophilic and hydrophobic domains that respectively accommodate polar (e.g. serine) and non-polar (e.g. leucine) amino acids as guest residues (MacEwan and Chilkoti, 2010). ELPs have excellent water solubility at low temperatures, however, they self-assemble into micelles at conditions above transition temperature (T_t). The T_t of elastin-like polypeptides is mainly determined by amino acid sequence (e.g. guest residue), molecular weight, ionic strength and protein concentration (Hassouneh et al., 2012; MacEwan and Chilkoti, 2010; McDaniel et al., 2014). ELPs can be designed as drug depots, which encapsulate hydrophobic drugs into the micelle cores during the transition phase when the temperature is increased above T_t; typically around 25 °C (Shi et al., 2013). Active targeting of cancer cells has been demonstrated by the attachment of specific receptor ligands to ELP micelle surfaces, such as Arg-Gly-Asp (RGD) (Simnick et al., 2010), asparagine-glycine-arginine (NGR) (Simnick et al., 2011) and cell penetrating peptide (Shi et al., 2013; Simnick et al., 2011).

Herein, we report the design and recombinant production of an ELP-GRP fusion protein. The formation of stable micelles was optimised and monitored using the hydrophobic and environment sensitive fluorescent dye 1,8-ANS (Hawe et al., 2008; Kim et al., 2010). An ELP without the C-terminal GRP (referred to ELP-C throughout this report) was produced to perform various control experiments. In addition to this, an ELP with three C-terminal lysine residues was designed (ELP-K) and used for the covalent linkage of a fluorescence marker. Subsequent formation of mixed micelles (e.g. ELP-GRP/ELP-K and ELP-C/ELP-K) was used for flow cytometry and fluorescence microscopy experiments, with GRPR expressing prostate cancer cell lines. The following experiments were carried out to prove functionality of the GRP displaying micelles: GRPR expressing PC-3 grade IV prostate adenocarcinoma cells were used to demonstrate specific receptor activation and calcium signalling by GRP decorated micelles. The specific interaction of fluorophore-labelled micelles with PC-3 and DU-145 cells was analysed by flow cytometry and GRP-labelled micelles, in contrast to non-labelled micelles, showed much stronger binding. Finally, GRP-mediated uptake into PC-3 cells was confirmed by confocal microscopy.

2. Materials and methods

2.1. Materials and reagents

The pET-31b(+) vector, DH5α™ and BL21(DE3) competent cells were purchased from Novagen Inc. (Madison, USA); NdeI, XhoI and T4 DNA Ligase were from New England Biolabs Ltd. (Genesearch Pty. Ltd., Arundel, Australia); QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit were purchased from Qiagen Pty. Ltd. (Victoria, Australia); BCA Protein Assay Kit was from Thermo Fisher Scientific (Adelaide, Australia); 4–20% Mini-PROTEAN® TGX™ Gels were from Bio-Rad Laboratories Pty. Ltd. (Gladesville, Australia); Polyethylenimine (PEI), 8-anilino-1-naphthalenesulfonic acid (1,8-ANS), RPMI-1640 medium, penicillin-streptomycin, trypsin and Atto 488 NHS ester were purchased from Sigma-Aldrich (Castle Hill, Australia). Fetal bovine serum (Interpath Services Pty. Ltd., Victoria, Australia); GRP peptide (Resolving IMAGES Pty. Ltd., Victoria, Australia); Fura-2 (Life Technologies Australia Pty. Ltd., Victoria, Australia). 8-well Lab-Tek chamber slides, with cover and permanox slide were purchased from Thermo Scientific (Victoria, Australia).

2.2. Plasmid vector design

The *ELP-C*, *ELP-GRP* and *ELP-K* genes were custom synthesised and cloned into the pMA plasmid vector using the NdeI and XhoI restriction sites (Thermo Fisher). Standard molecular biology techniques were applied for sub-cloning of the ELP genes into the bacterial expression vector pET-31b (+). Briefly, the *ELP-C*, *ELP-GRP* and *ELP-K* genes were cut from the pMA vector with an NdeI/XhoI co-digest, followed by agarose gel purification (gel extraction and PCR product purification kit, Qiagen) prior to ligation with T4 ligase into the NdeI/XhoI treated pET-31b(+) vector.

2.3. Expression and purification of ELP-C, ELP-K and ELP-GRP proteins

BL21(DE3) *E. coli* cells were transformed with either pET-*ELP-C*, pET-*ELP-K* or pET-*ELP-GRP* (Table 1). Transformed *E. coli* bacteria were cultured in 400 mL LB medium with 50 μg/mL ampicillin on

Table 1
Peptides evaluated in this study.

Name	Nomenclature	Amino acid sequence	T_t (°C)
ELP-C	I48S48	$G(VPGIG)_{48}(VPGSG)_{48}Y$	27.0
ELP-GRP	I48S48-H-G	$G(VPGIG)_{48}(VPGSG)_{48}Y$-HHHHHH-GNHWAVGHLM	22.0
ELP-K	I48S48-K	$G(VPGIG)_{48}(VPGSG)_{48}Y$-KGGKGGK	24.0